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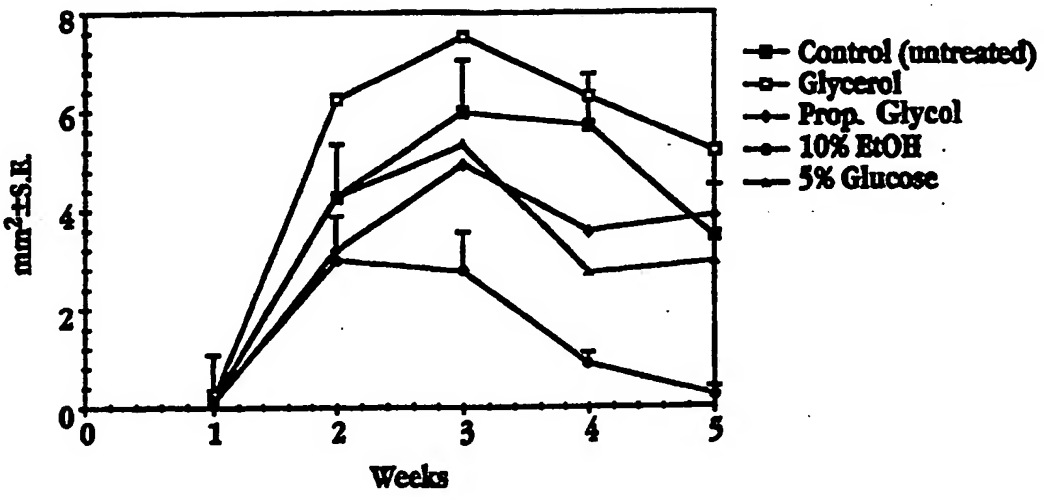

(57) Abstract

Compositions and methods for topical treatment of fungal or protozoal infections of the skin or nails are described. The compositions include a lipid formulation of amphotericin B which is dispersed in an aqueous carrier containing about 2 % to about 35 % by volume ethanol relative to water.

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FORMULATIONS FOR TOPICAL TREATMENT OF SKIN INFECTIONS

Field of the Invention

The present invention relates to topical formulations of antimicrobial agents. In particular, the invention is directed to the use of topical formulations of amphotericin B against parasites and infectious organisms, and to the topical use of synergistic combinations of amphotericin B with other antifungal agents.

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Background of the Invention

Amphotericin B (AmB), a polyene antibiotic, is a potent antifungal and antiparasitic agent

having a broad spectrum of activity, against organisms having ergosterol in their membranes. It is commonly used as a systemic agent, administered intravenously, against such infections as aspergillosis, systemic candidiasis, cryptococcosis, North American blastomycosis, coccidioidomycosis, histoplasmosis, zygomycosis, and sporotrichosis. It has also shown high activity against leishmania infection. However, because amphotericin B can be highly toxic, particularly nephrotoxic, when given systemically, it is thus generally used only after other treatments have failed (*e.g.* Giri, 1993).

Toxic side effects are typically greatly reduced when a drug is administered topically, since much lower doses can generally be used, and a very small amount of the drug is absorbed systemically. The use of topical amphotericin B preparations on mucous membrane surfaces, such as those of the eye, mouth and respiratory and digestive passages, often in combination with other agents, has been described (*e.g.* Lotery *et al.*, 1994; Pleyer *et al.*, 1992; Aerdts *et al.*, 1991; Bent *et al.*, 1996).

The skin, however, is a very effective barrier for substances with a molecular weight higher than a few hundred daltons. For example, topical treatment of Cutaneous leishmaniasis (CL), a widespread and potentially disfiguring protozoal infection of the skin, with paromomycin, an aminoglycoside antibiotic, has been shown to be effective in some cases (El-On *et al.*, 1985) but not in others (Bensalah *et al.*, 1995; Klaus *et al.*, 1994; Neva *et al.*, 1997). The currently available topical treatment for CL is a formulation of paromomycin and methylbenzethonium chloride. This preparation also has the disadvantages of significant initial irritation and high cost. Cutaneous *Leishmania major* lesions in mice were also unresponsive to a topical preparation of AmB in soft white paraffin containing 12% methylbenzethonium chloride (El-On *et al.*, 1984), although the drug is effective against leishmaniasis when administered systemically. In order to obtain effective drug penetration through the skin, therefore, an effective drug-carrier system is required.

Summary of the Invention

The present invention includes, in one aspect, a composition and method for topical treatment of a fungal or protozoal infection of the skin or nails. The composition includes a therapeutic amount of a lipid formulation of amphotericin B, dispersed in an aqueous carrier containing about 2% to about 35% by volume ethanol relative to water. In accordance with the method of the invention, a therapeutic amount of such a composition is administered topically to the site of infection in a subject in need of such treatment.

Preferably, the aqueous carrier is about 5% to 25% by volume ethanol relative to water; more preferably, the formulation is glucose-free. The lipid-based formulation is preferably a stable

complex of amphotericin B with at least one amphipathic lipid molecule. It may also be a liposomal formulation, comprising amphotericin B encapsulated in liposomes composed of vesicle-forming lipids.

In either type of formulation, the lipids are preferably phospholipids, glycolipids, sterols, or sterol derivatives, or a combination thereof, having a high affinity for amphotericin B. Particularly preferred lipids are selected from dimyristoyl phosphatidyl choline (DMPC), dimyristoyl phosphatidyl glycerol (DMPG), and distearoyl phosphatidyl glycerol (DSPG), soy phosphatidyl choline, egg phosphatidyl choline, cholesterol, and cholesteryl esters, such as cholesteryl sulfate. Cholesteryl sulfate is particularly preferred.

In a preferred embodiment of the method, the fungal or protozoal infection being treated is present in a human subject. The topical infection may be, for example, cutaneous leishmaniasis, cutaneous candidiasis, candida paronychia or candida onychomycosis infection. In a preferred embodiment of the method, the infection being treated is cutaneous leishmaniasis.

For treatment of dermatophyte infections, the composition further includes a second drug with anti-dermatophyte activity, and the method of the invention further comprises administering such a second drug with the above-described lipid formulation of amphotericin B. The dermatophyte infection being treated is preferably a tinea or onychomycosis infection. The second drug is preferably griseofulvin, allicin, a polyoxin, a nikkomycin, or fluorocytosine, and is most preferably griseofulvin or allicin. Preferably, the combined composition has potentiated anti-dermatophyte activity relative to the combined activity of compositions containing either amphotericin B or the second drug alone.

Also contemplated are a composition and method for topical treatment of a fungal or protozoal infection of the skin or nails, where the composition includes a therapeutic amount of allicin dispersed in an aqueous carrier containing about 2% to about 35% by volume ethanol relative to water. In accordance with the method of the invention, a therapeutic amount of such a composition is administered topically to the site of infection in a subject in need of such treatment. Preferably, the aqueous carrier is about 5% to 25% by volume ethanol relative to water.

These and other objects and features of the invention will become more fully apparent when the following detailed description of the invention is read in conjunction with the accompanying drawings.

Brief Description of the Drawings

Figure 1 shows the effect of formulations of amphotericin B/cholesteryl sulfate (Amphocil®) in various solvents, applied topically, on the size of cutaneous leishmaniasis lesions in CBA inbred

mice;

Figure 2 shows the effect of Amphocil® in aqueous ethanol and of aqueous ethanol alone on CL lesion size, as in Figure 1;

Figure 3 shows the effect of Amphocil® in aqueous ethanol and in distilled water, and of Fungizone® in aqueous ethanol, on CL lesion size, as in Figures 1-2; and

Figures 4 and 5 are graphic representations of selected data from Tables 5 and 6, respectively, showing levels of AmB measured in the skin and internal organs after topical administration of AmB/lipid complexes in aqueous ethanol.

Detailed Description of the Invention

I. Definitions

The terms below have the following meanings unless indicated otherwise.

An "amphipathic lipid molecule" refers to a molecule of a lipid, that is, any of a group of hydrophobic substances found in nature and having aliphatic hydrocarbon chains, *e.g.* fatty acids, sterols, phospholipids, and various derivatives thereof, having a hydrophobic region and a polar, hydrophilic region. These include "vesicle-forming lipids", which can form bilayer vesicles in water, or which can be stably incorporated into a lipid bilayer, with the hydrophobic moiety in contact with the interior, hydrophobic region of the bilayer membrane, and the polar head group moiety oriented toward the exterior, polar surface of the membrane. Vesicle-forming lipids of this type typically include one or two hydrophobic acyl hydrocarbon chains or a steroid group and may contain a chemically reactive group, such as an amine, acid, ester, aldehyde or alcohol, at the polar head group. Included in this class are the phospholipids, such as phosphatidyl choline (PC), phosphatidyl glycerol (PG), phosphatidyl ethanolamine (PE), phosphatidic acid (PA), phosphatidyl inositol (PI), and sphingomyelin (SM), where the two hydrocarbon chains are typically between about 14-22 carbon atoms in length, and have varying degrees of unsaturation. Specific examples are dimyristoyl phosphatidyl choline (DMPC), dimyristoyl phosphatidyl glycerol (DMPG), and distearoyl phosphatidyl glycerol (DSPG). Other vesicle-forming lipids include sterols, such as cholesterol.

A "stable complex" of amphotericin B with at least one amphipathic lipid molecule is a complex in which the lipid molecule has a high binding affinity for amphotericin B. Preferably, the affinity is at least comparable to that of cholesteryl sulfate. Binding, or lack thereof, of AmB to a lipid or mixture of lipids may be evaluated by spectroscopic or other methods, as discussed in Section IIA below.

II. Topical Amphotericin B Formulations and Effect on Cutaneous Leishmaniasis Lesions

A. Lipid Components

Various amphipathic phospholipids, as described above, as well as sterols and sterol derivatives, may be used in the current compositions. Of the latter, polar cholesteryl esters, such as cholesteryl sulfate, are a preferred group. In general, compounds having a high affinity for AmB are most effective in the topical formulations described herein. For example, in Amphocil®, as discussed below, the lipid component, cholesteryl stearate, has a much higher AmB association constant than sodium deoxycholate, used in Fungizone® (Cohen *et al.*, 1996).

The binding affinity of a lipid for AmB may be measured by various methods reported in the literature, many of which are based on changes in the absorption spectra of AmB upon complexation. See, for example, Janoff *et al.*, 1991; Readio *et al.* (1982) and references therein. Circular dichroism spectroscopy (Jullien *et al.*, 1988; Milhaud *et al.*, 1994) has been reported to be more accurate in systems with higher concentrations of AmB. The interaction of AmB with lipid vesicles may also be evaluated by observing leakage of marker substances from such vesicles (*e.g.* carboxyfluorescein, as reported by Milhaud *et al.*, 1994).

B. Preparation of AmB/Cholesteryl Sulfate Formulations

The amphotericin B formulations used to produce the results shown in Figs. 1-3 were prepared using Amphocil®, a complex of amphotericin B and cholesteryl sulfate, available as a colloidal dispersion from Sequus Pharmaceuticals. This preparation, normally administered by injection, contains small (approximately 100 nm) diskoid particles (see *e.g.* Barenholz and Cohen, 1995) composed of 1:1 (molar ratio) amphotericin B and cholesteryl sulfate. The commercial preparation also contains glucose (5%), as do most drug formulations intended for i.v. injection.

Dispersions of Amphocil® were prepared in the following media:

A1. 5% ethanol in DDW (double distilled water)

A2. 10% ethanol in DDW

A3. 25% ethanol in DDW

B. 5% glucose in DDW

C. 100% propylene glycol

D. 100% glycerol

E. 1:1 glycerol:DDW

Amphotericin B itself is insoluble in water or ethanol; however, Amphocil® disperses easily in

aqueous solutions of 5% glucose or 5% ethanol. To obtain a dispersion in propylene glycol or glycerol, strong vortexing and ultrasonic irradiation were necessary. The amount of amphotericin B was quantified using HPLC followed by absorbance at 408 nm, and brought to a concentration of 5 mg/ml for particle size measurement.

A glucose-free preparation, used in the clinical trial described in Section D, was prepared by diluting Amphocil® with water and dialyzing the solution (cellophane dialysis tubing, molecular weight cutoff 12-14,000 daltons) against double distilled water for 24 hours, before addition of ethanol.

Particle size distribution of formulations A and B, above, was measured by photon correlation spectroscopy, giving the results shown below.

Table 1

Formulation	Amount (%)	Size (nm)	S.E.
A1	2 90 8	18 167 822	3 53 96
A2	80 20	205 1270	83 150
A3	90 10	259 1260	81 150
B	97 3	7.3 104	1 23

Formulations C, D and E, which dispersed with difficulty, formed particles which were too large to be measured by photon correlation spectroscopy. These preparations were measured with a Coulter multisizer, which measures particle size from 0.8-30 μm.

Table 2

Formulation	Particle Concentration	Size (Median)	Size (Mean)	S.E.
C	1.2×10^7/ml	15.7 μm	12.5 μm	8.51
D	5.6×10^9/ml	1.38 μm	3.75 μm	4.6
E	4.8×10^9/ml	1.5 μm	4.4 μm	5.46

C. Therapeutic Effect of Aqueous Ethanolic AmB/Cholesteryl Sulfate on Cutaneous *Leishmaniasis* Infection *in vivo*: Mouse Model

CBA inbred mice, which develop self healing lesions, were used for the *in vivo* experiments. Each mouse was injected in the base of the tail with 5×10^6 promastigotes from a stationary (infective) growth culture. These parasites were found to be sensitive to aqueous formulations *in vitro* (see below). The mice were divided into groups of at least 8 mice each, with one group as an untreated control. The topical preparations used were dispersions of Amphocil® in glycerol, propylene glycol, 10% EtOH in DDW, and 5% glucose in DDW, as described above.

From 24 hours after injection onwards, and for the next 4 weeks, 10 µl of each preparation (concentration 2 mg/ml) was applied daily on the base of the tail. Each mouse received a total of 15 drug applications, representing approximately 300 µg amphotericin B.

Lesion size was measured weekly. To determine the diameter, the average was taken between the longest distance across the lesion and the length of the line bisecting this distance at a 90° angle.

Results are shown in Fig. 1. The Figure shows that topical administration of Amphocil® in 10% ethanol caused a marked reduction in lesion size. The effect was statistically significant from week 3 onwards. The group receiving Amphocil® in 5% glucose had statistically smaller lesions than the controls at only one timepoint (week 4), and the other groups showed little or no effect.

No apparent correlation was noted between particle size, as measured above, and therapeutic effectiveness of a given dispersion. Additional experiments showed that dispersions of amphotericin B and cholesteryl sulfate in 5%, 10% and 25% ethanol were approximately equivalent in their effect on lesion size. Aqueous ethanol alone, applied at 5%, 10% and 25% to three experimental groups, did not affect lesion size (Fig. 2); nor did a suspension of amphotericin B and cholesteryl sulfate in water (Fig. 3).

The above results suggest that the therapeutic efficacy of amphotericin B/lipid formulations in ethanolic aqueous carriers is related to the presence of low concentrations of ethanol in the aqueous carrier, which is believed to enhance skin permeability and penetrability of the drug.

D. Clinical Trial in Human Patients

D1. Study Protocol

Patients were 18 years or older, and had 3-10 CL lesions (average 5 lesions), mostly on the arms and legs, but were otherwise healthy. Subjects were excluded if they were pregnant or breast feeding, undergoing other specific treatments for leishmaniasis, or afflicted with a significant underlying disease. The study was approved by the Hadassah University Hospital Human Rights

(Helsinki) Committee.

Prior to the study, the clinical diagnosis of CL was confirmed for each patient by a direct smear stained by Giemsa. Kidney function tests and serology for HIV and hepatitis B and C were performed. A detailed history and physical examination were done and lesion dimensions were measured. To determine the diameter, the average was taken between the longest distance across the lesion and the length of the line bisecting this distance at a 90° angle. From this value, the lesion surface was calculated.

Each patient was given two vials, one containing Amphocil® dispersed in a 5% ethanol solution and the other containing 5% ethanol in water. The location of the lesions was designated on respective bottles (e.g. bottle #1: right leg and arm; bottle #2: left shin and knee). The patient was instructed to apply 2-5 drops from the designated bottles onto each lesion three times daily. The first 5 patients were treated with a glucose (5%)-containing AmB preparation, and the next 6 patients were treated with a glucose-free preparation. Each treated lesion received 1.5-3.75 mg amphotericin B per day. Patients were followed up every 1-2 weeks in the course of the treatment.

D2. Materials

Amphocil®, as described above, was dissolved in 5% ethanol : 95% distilled water to a concentration of 5 mg/ml AmB. This preparation contained 5% glucose. In order to obtain a glucose-free preparation, water was added to Amphocil®, and the solution was dialyzed (cellophane dialysis tubing, molecular weight cut off 12-14000 daltons) against double distilled water for 24 hours, before addition of ethanol. Size distribution and mean particle size of the dispersion were compared before and after dialysis by photon correlation spectroscopy using Coulter submicron particle analysis (Coulter N4SD submicron particle analyzer, Coulter Electronics Ltd, Luton, England). Size distribution was unimodal before and after dialysis, although mean size increased slightly, from 104±23 nm before dialysis to 129±29 nm after dialysis. After dialysis, particle size remained stable for at least 5 weeks when stored at 4 °C.

D3. Results

Lesion diameters were measured every 7-14 days in the course of the treatment. Lesion area (mm^2) was determined. and the difference before and after treatment (Δmm^2) was calculated. The nonparametric Wilcoxon Matched-Pairs Signed-Ranks Test was used for statistical analysis. The SPSS program was used to run the analysis.

Eleven of the thirteen patients enrolled in the study completed the trial protocol. For each patient, the average lesion area for drug and for placebo treated lesions was calculated before and

after treatment. The results, summarized in Table 3, show that in the patients treated with the ethanolic glucose-containing preparation, only patient 1, who had open wounds, showed a significant difference in reduction of lesion area between the drug- and the placebo-treated lesions. Two other patients showed moderate benefits. However, in the group that received the ethanolic glucose-free preparation, a significant improvement was demonstrated in the Amphocil® treated lesions, compared to the placebo treated lesions, for all patients (2-Tailed p=0.027). On follow-up visits, complete healing of the lesions was observed, with no evidence of relapse.

Each treated lesion received 1.5-3.75 mg amphotericin B per day. There were neither local or systemic side effects observed, nor was amphotericin-B detected in patient serum, as assessed by high pressure liquid chromatography (HPLC).

Table 3: Effect of Ethanolic Amphocil® on SCL Lesions in Humans

				Vehicle		Ethanolic Amphocil®	
patient no.	glucose	t_x time (days)	lesion age (months)	no. of lesions treated	Δ mm^2 (%decrease)	no. of lesions treated	Δ mm^2 (%decrease)
1	+	21	3	2	0 (0)	1	- 84 (87)
2	+	14	1	2	+7.6 (i24)	2	+0.6 (i6)
3	+	30	1	1	0 (0)	2	0 (0)
4	+	46	3	1	+10.5 (i47)	1	+21.9 (i70)
5	+	28	3	2	+47.3 (i59)	2	+25.2 (i49)
6	-	22	4	1	- 4 (25)	1	- 22.1(56)
7	-	24	3	4	- 34 (9)	9	- 107 (50)
8	-	64	4	1	+8 (i27)	2	- 7.8 (100)
9	-	35	3	1	- 2 (15)	2	- 4.2 (34)
10	-	10	2	2	+31.4 (i93)	2	+14.8 (i39)
11	-	14	1	2	4.2 (56)	4	8.7 (78)

Δmm^2: lesion size after treatment - lesion size before treatment; i: % increase in lesion size.

This study has demonstrated that ethanolic glucose-free Amphocil®, used in very small amounts, causes a significant improvement of CL lesions in human subjects. The therapeutic benefits of the preparation may be due to its physical properties. The use of Amphocil®, having a lipid component with a high AmB association constant, allows for effective penetration into the hydrophobic layers of the skin. The addition of ethanol causes an increase in particle size, as shown in Table 1. This may cause the less flexible AmB to protrude from the assembly and become more readily available to the skin.

This study also demonstrates that the glucose present in the original Amphocil® vial (5%)

markedly reduces the beneficial therapeutic effect of the topically applied compound. The precise inhibitory mechanism of glucose is not yet clear. It may be related to the high viscosity introduced by glucose, or to changes induced by glucose on the lipid/water interface of colloidal particles (Crowe *et al.*, 1988).

E. Other AmB/Lipid Formulations

Other lipid formulations of amphotericin B may also be used, preferably complexes of amphotericin B with amphipathic lipid molecules having a high affinity for the drug, as well as dispersions of liposomes in which the drug has been encapsulated, according to well known methods.

For example, ABPLC (amphotericin B phospholipid complex, described in Engelhard *et al.*, 1993) is a 1:3 molar complex of AmB with a 7:3 molar mixture of DMPC and DMPG. This complex tends to form large (approx. 2.75 μm) aggregate particles (Barenholz and Cohen, 1995). A topical formulation of ABPLC in 0.9% NaCl/5% ethanol was found to be at least as effective as the AmB/cholesteryl sulfate formulations in reducing lesion size, in spite of the large particle size in this formulation. Thus, different types of AmB/lipid assemblies have been found to be effective.

The commercial AmB preparation Fungizone® (Bristol Myers Squibb) contains sodium deoxycholate, which has relatively low affinity for the drug (see *e.g.* Brajtburg and Bolard, 1996; Janoff *et al.*, 1991). Accordingly, ethanolic formulations containing Fungizone® were much less effective topically than those described above (Fig. 3), although they were highly effective *in vitro* (see Section III below).

F. Dosages

As demonstrated by the biodistribution studies described below, dosages of AmB administered topically, according to the method of the invention, result in much lower levels of the drug in internal organs than lower dosages administered systemically. Accordingly, higher dosages may be used with greatly reduced toxic side effects.

For treatment of a topical fungal or protozoal infection, an effective amount of an aqueous ethanolic solution or dispersion of a lipid/AmB complex, such as described herein, is applied to the site of the infection, preferably about once daily. Preferably, the formulation is glucose-free. A single application preferably contains about 5 to 500 μg, and more preferably about 20 to 200 μg, of amphotericin B, depending on the severity of the infection. Applications are repeated as necessary until the infection is substantially reduced or eliminated.

III. Effect of Amphotericin B Formulations on *Leishmania Major* Promastigotes *in vitro*

To further elucidate the mode of action of the present formulations, their effect in cell cultures, relative to that of Fungizone®, was tested as follows. *L. major* promastigotes (5×10^6/ml) were incubated at 28°C in RPMI-1640 tissue culture medium containing 20% fetal calf serum. Amphocil® formulations in aqueous EtOH or DDW (see table below) were added, and the number of viable (moving) parasites was counted after 24 hours. The results obtained were expressed as percent death of parasites in treated cultures (100 - # viable parasites in treated cultures/ # viable parasites in untreated cultures). Each value is the average of 2 independent experiments ± s.e.

Table 4: Percent Death of Parasites *in vitro* (Avg. ± S.E.)

[μm]	5% EtOH	10% EtOH	25% EtOH	DDW
1	99 ± 2	98 ± 2	97 ± 4	99 ± 1
0.3	78 ± 13	62 ± 25	65 ± 31	81 ± 5
0.1	25 ± 25	33 ± 13	35 ± 11	21 ± 15
0.3	11 ± 11	0	6 ± 6	3 ± 3
AmB/deoxycholate (Fungizone®)				
1	100 ± 0			
0.1	96 ± 4			
0.01	85			
0.001	6			

Amphotericin B/deoxycholate (Fungizone®) in 5% ethanol, although highly effective in killing parasites *in vitro* (see Table 4), was ineffective *in vivo* when applied topically (Fig. 3). As noted above, the deoxycholate carrier molecule has a relatively low affinity for AmB.

The results further show that ethanol is not necessary for effectiveness of amphotericin B *in vitro*, since the same effect was obtained with AmB/cholesteryl sulfate prepared in DDW or in different aqueous ethanolic solutions. These results demonstrate that the addition of ethanol does not affect the parasites directly, and thus probably acts by facilitating the transport of the drug through the skin.

Application of the ethanolic formulation described herein to other antibacterial and antifungal drugs is also contemplated. In particular, the compound allicin, a component of garlic, has long been known to show such activity *in vitro* or when administered systemically. Preliminary studies suggest that it has excellent potential in topical ethanolic formulations, where the compound is dissolved or dispersed in an aqueous carrier containing about 2% to about 35% by volume ethanol relative to water.

IV. Biodistribution of Amphotericin B Formulations after Topical and Systemic Administration

Table 5 shows the level of AmB detected in body tissues (μg/g) after topical administration of various levels of Amphocil® in 10% EtOH, as described above. Selected data from the table are also represented graphically in Figure 4.

Once daily for 15 days, 30 μl of solution containing the amount of AmB shown in the first column was applied topically, giving the cumulative totals shown in the second column. Levels in the tissue were measured one day after administration was terminated. The level in the skin (at the site of application) was also measured one hour after administration was terminated.

Table 5: Biodistribution of Topically Applied Ethanolic Amphocil®

AMB (μg/application; total dose*)	μg/g tissue (% of total dose)				
	skin: 1 hr.#	Skin: 24 hrs.	Kidney	Liver	Spleen
0 (control); 0	0	0	0	0	0
180 μg/day; 2.7 mg	180 (6.6)	0	0	0	1 (0.04)
540 μg/day; 8.1 mg	725 (8.9)	12 (0.15)	2.2 (0.03)	1.9 (0.02)	5.5 (0.07)
1620 μg/day; 24.3 mg	1500 (6.2)	42 (0.17)	36 (0.15)	6.4 (0.03)	11.5 (0.05)

*Total of 15 applications.
#Skin: only area of application was sampled.

No amphotericin B was detected in the kidneys and liver by HPLC (detection level: 0.05 μg), and only a small amount in the spleen, after total dosages of 0.9 and 1.8 mg of the drug. The amount detected in the skin was 138 μg/g tissue one hour after the last application, and 18 μg/g after one day. Low levels were detected in the other tissues after total dosages of 8.1 mg and 24.3 mg, but still many times less than the effective level in the skin. Toxic side effects commonly associated with amphotericin B, particularly to the kidney, should thus be greatly diminished or absent while still providing effective dosages to the site of infection. Also indicated, for comparison with the data below, are the approximate percentages of the total dose of AmB detected per gram of each tissue.

Table 6 shows a similar study using topical ethanolic ABPLC (amphotericin B phospholipid complex, described above), administered daily for 15 days as above. Cumulative total dosages are given in the second column, and levels in the tissue, as above, in columns 3-6. Selected data from the table are also represented graphically in Figure 5. Although this formulation was found to be effective topically against CL lesions, the data shows that the biodistribution is not as favorable as

shown for the topical ethanolic Amphocil® formulation, above.

Table 6: Biodistribution of Topically Applied Ethanolic ABPLC

AmB (μg/ application)	AmB (total dose)	μg/g of tissue (% of total)			
		Skin	Kidney	Liver	Spleen
0 (control)	0.00	0.00	0.00	0.00	0.00
20 μg/day	0.30 mg	6.10 (2.0)	4.50 (1.5)	2.10 (0.70)	1.40 (0.47)
60 μg/day	0.90 mg	20.4 (2.3)	4.40 (0.49)	3.60 (0.40)	4.00 (0.44)

Table 7 shows the biodistribution of AmB after intravenous administration of Amphocil®, ABPLC, and Fungizone®. The data show that, even for much smaller cumulative dosages (40 to 100 μg, vs. one to several mg for topical application), comparable or sometimes greater levels of drug were detected in the internal tissues. For Amphocil®, in particular, large amounts of the drug were found in the liver and spleen.

Table 7: Biodistribution of I.V. Administered AmB Formulations

Formulation	Total Dose[1]	Kidney		Liver		Spleen	
		μg/g Tissue[2]	μg/organ	μg/g Tissue[2]	μg/organ	μg/g Tissue[2]	μg/organ
Fungizone®	40 μg	3.3 (8.3)	0.4	4.2 (10.5)	5.86	3.07 (7.7)	0.47
Amphocil®	100 μg	0.45 (0.45)	0.173	48.4 (48)	67.9	13.6 (14)	2.18
ABPLC	100 μg	0.68 (0.68)	0.26	3.08 (3.1)	4.32	4.36 (4.4)	0.68

[1] Five i.v. injections to 20g mice of 0.4 μg/g (8 μg) Fungizone® or 1 μg/g (20 μg) Amphocil® or ABPLC

[2] Figure in parenthesis = percent of total dose

V. Combined Drug Treatment for Dermatophytes

Amphotericin B, although a very effective antifungal agent, is much less active against

dermatophytes. Amphotericin B acts by binding to ergosterol in the cell membrane, which is also present in dermatophytes, resulting in an increase in membrane permeability and leakage of intracellular components. One possible explanation for its decreased activity in dermatophytes is the presence of larger amounts of chitin-containing cell wall of these fungi.

The activity of AmB against dermatophytes may be enhanced by the addition of drugs which act against these organisms by a different mechanism. For example, griseofulvin, an effective drug against dermatophytes, inhibits cell division by interfering with the structure and function of microtubules, and is also believed to affect cell wall synthesis. It is widely considered the drug of choice for the treatment of *tinea capitis*, especially *Microsporum* species. Polyoxins and nikkomycins are inhibitors of chitin synthetase and have been shown to be effective against certain dermatophytes, although their spectrum of activity appears to be more restricted than that of griseofulvin. Fluorocytosine is believed to act via the replacement of uracil with 5-fluorouracil in the RNA of the organism.

The inhibitory effect of AmB and griseofulvin on the growth of *Trychophyton* mentagrophytes was tested in solid-phase culture. The results showed that 2 ug/ml griseofulvin and 1 mg/ml Fungizone® (a micellar formulation containing sodium deoxycholate; Bristol Myers Squibb), when used separately, affected fungal growth only minimally (reduction in radius about 1 mm in each case). Both drugs used in combination, however, had a significant inhibitory effect (9 mm). The combination thus showed a significantly potentiated antifungal effect relative to the combined effects of each agent used alone.

While the invention has been described with reference to specific methods and embodiments, it will be appreciated that various modifications may be made without departing from the invention.

IT IS CLAIMED:

1. A composition for topical treatment of a fungal or protozoal infection of the skin or nails, comprising a lipid-based formulation of amphotericin B, dispersed in an aqueous carrier containing about 2% to about 35% by volume ethanol relative to water.

2. The composition of claim 1, wherein said lipid-based formulation comprises a stable complex of amphotericin B with at least one amphipathic lipid molecule.

3. The composition of claim 2, wherein said at least one amphipathic lipid molecule is a phospholipid, a glycolipid, a sterol, a sterol derivative, or a combination thereof.

4. The composition of claim 3, wherein said lipid molecule is selected from the group consisting of dimyristoyl phosphatidyl choline, dimyristoyl phosphatidyl glycerol, distearoyl phosphatidyl glycerol, soy phosphatidyl choline, egg phosphatidyl choline, cholesterol, and cholesteryl sulfate.

5. The composition of claim 4, wherein said lipid molecule is cholesteryl sulfate.

6. The composition of claim 5, wherein said formulation comprises an approximately 1:1 molar ratio of amphotericin B to cholesteryl sulfate.

7. The composition of claim 1, wherein said aqueous carrier contains about 5% to about 25% ethanol relative to water.

8. The composition of claim 1, wherein said formulation is glucose-free.

9. The composition of claim 1, useful in treating a dermatophyte infection, further comprising a second drug with anti-dermatophyte activity.

10. The composition of claim 9, wherein said second drug is selected from the group consisting of allicin, griseofulvin, a polyoxin, a nikkomycin, and fluorocytosine.

11. The composition of claim 9, wherein the composition has potentiated anti-dermatophyte activity relative to the combined activity of compositions containing either amphotericin B or said

second drug alone.

12. A composition for topical treatment of a fungal or protozoal infection of the skin or nails, comprising a therapeutic amount of allicin dispersed in an aqueous carrier containing about 2% to about 35% by volume ethanol relative to water.

13. The composition of claim 12, wherein said aqueous carrier contains about 5% to about 25% ethanol relative to water.

14. A method of treating a fungal or protozoal infection of the skin or nails, comprising administering topically to the site of the infection a therapeutic amount of a lipid-based formulation of amphotericin B, dispersed in an aqueous carrier containing about 2% to about 35% by volume ethanol relative to water.

15. The method of claim 14, wherein said lipid-based formulation comprises a stable complex of amphotericin B with at least one amphipathic lipid molecule.

16. The method of claim 15, wherein said at least one amphipathic lipid molecule is a phospholipid, a glycolipid, a sterol, a sterol derivative, or a combination thereof.

17. The method of claim 14, wherein said infection is present in a human subject.

18. The method of claim 16, wherein said lipid molecule is cholesteryl sulfate.

19. The method of claim 18, wherein said formulation comprises an approximately 1:1 molar ratio of amphotericin B to cholesteryl sulfate.

20. The method of claim 14, wherein said aqueous carrier contains about 5% to about 25% ethanol relative to water.

21. The method of claim 14, wherein said formulation is glucose-free.

22. The method of claim 14, wherein said infection is cutaneous leishmaniasis or cutaneous candidiasis.

23. The method of claim 22, wherein said infection is cutaneous leishmaniasis.

24. The method of claim 14, wherein said fungal or protozoal infection is present in a human subject.

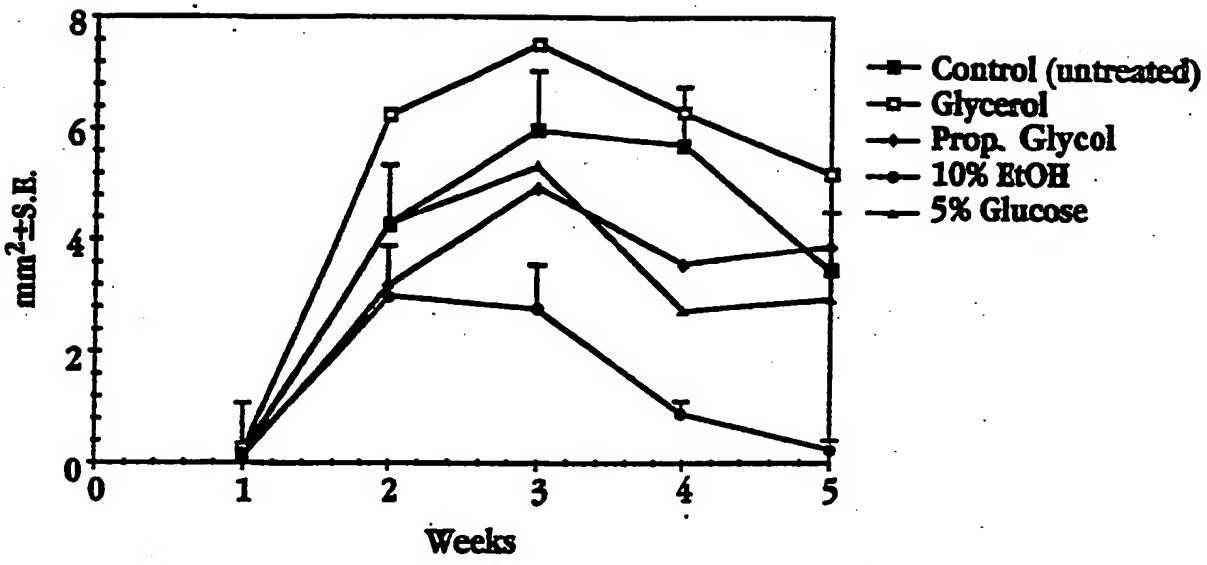
25. The method of claim 14, wherein said infection is a dermatophyte infection, and further comprising administering with said lipid-based formulation of amphotericin B, a second drug with anti-dermatophyte activity.

26. The method of claim 25, wherein said second drug is selected from the group consisting of allicin, griseofulvin, a polyoxin, a nikkomycin, and fluorocytosine.

27. The method of claim 25, wherein said administering produces potentiated anti-dermatophyte activity relative to the combined effects of administering amphotericin B and said second drug individually.

28. A method of treating a fungal or protozoal infection of the skin or nails, comprising administering topically to the site of the infection a therapeutic amount of allicin dispersed in an aqueous carrier containing about 2% to about 35% by volume ethanol relative to water.

29. The method of claim 28, wherein said aqueous carrier contains about 5% to about 25% ethanol relative to water.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0
1
2
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Weeks
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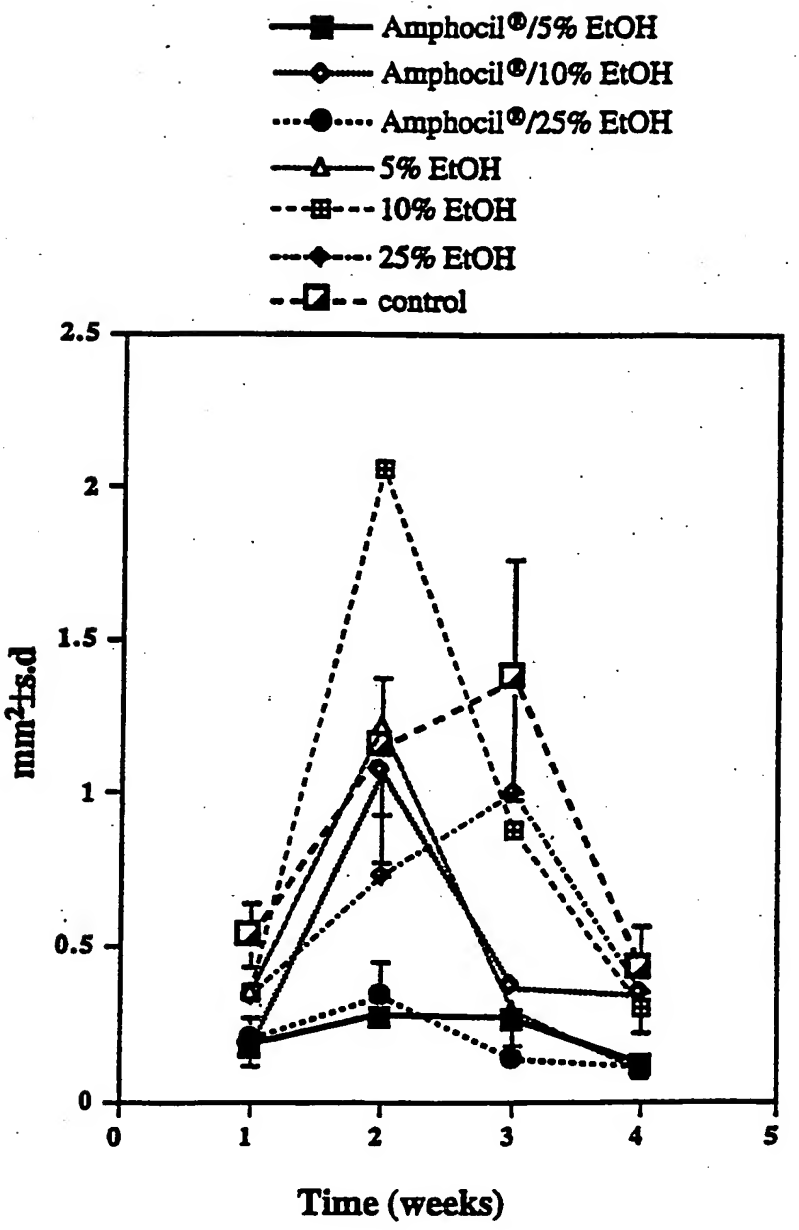
Glycerol
Prop. Glycol
10% EtOH
5% Glucose

Fig. 1


Amphocil®/5% EtOH
Amphocil®/10% EtOH
Amphocil®/25% EtOH
5% EtOH
10% EtOH
25% EtOH
control
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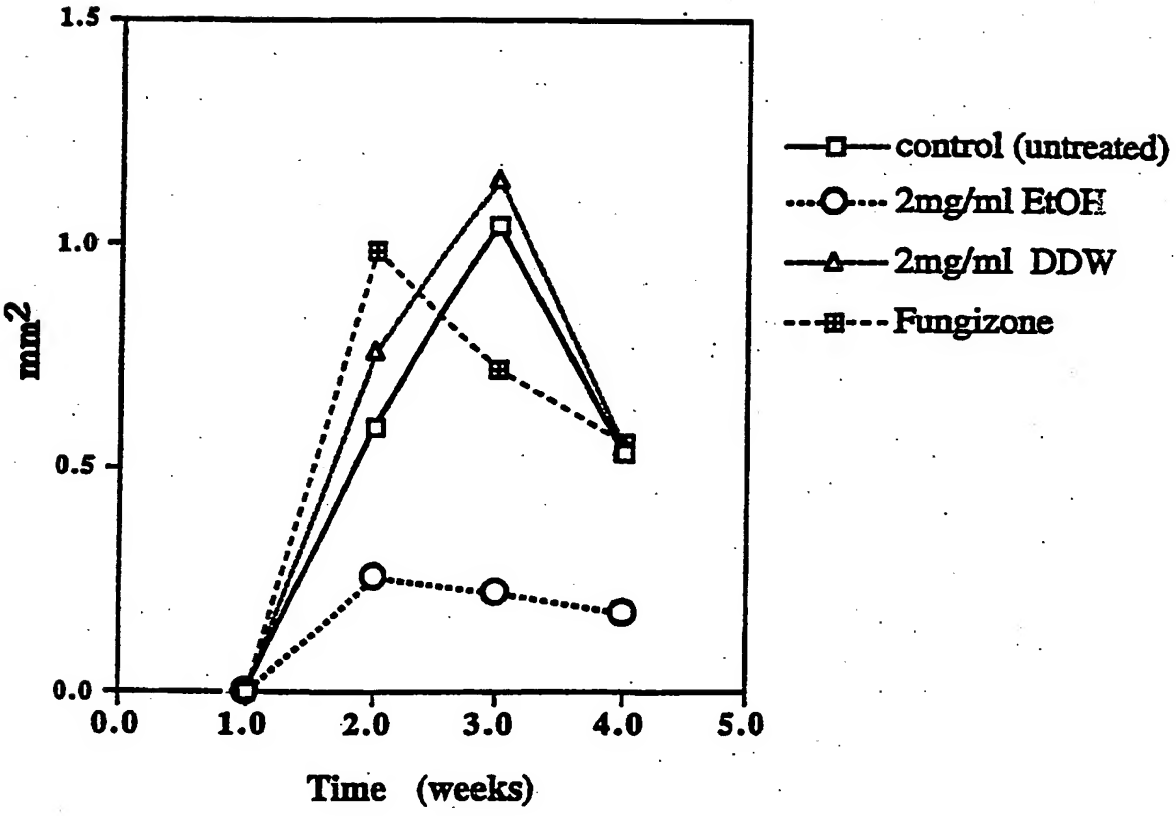
2
1.5
1
0.5
0
mm²±s.d
0
1
2
3
4
5
Time (weeks)

Fig. 2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1.0
0.5
0.0
mm2
0.0
1.0
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Time (weeks)
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2mg/ml EtOH
2mg/ml DDW
Fungizone

Fig. 3

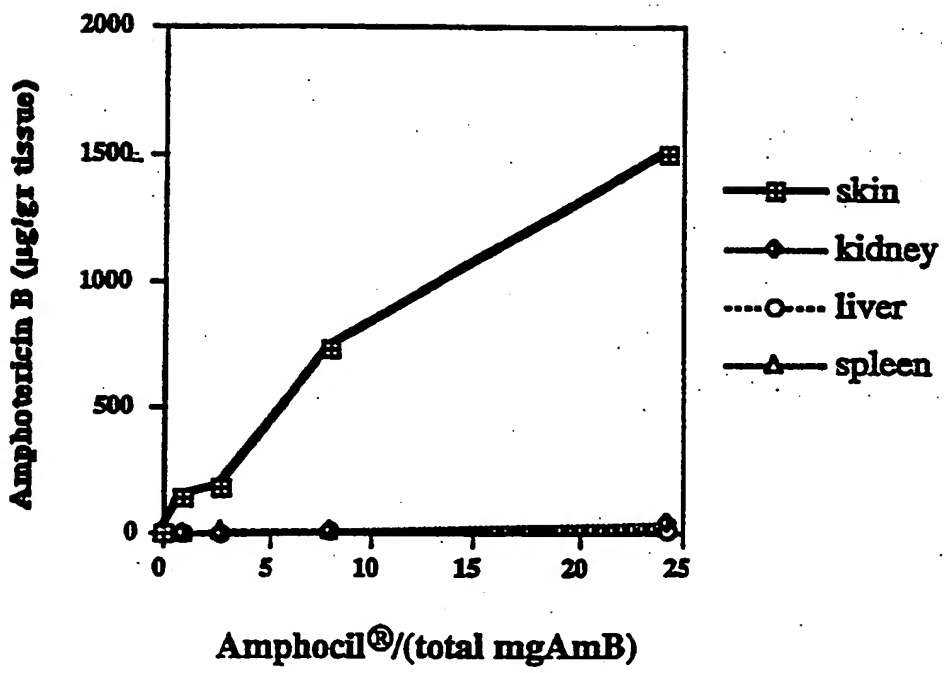
2000
1500
1000
500
0
Amphotericin B (µg/gr tissue)
0
5
10
15
20
25
Amphocil®/(total mgAmB)
skin
kidney
liver
spleen

Fig. 4

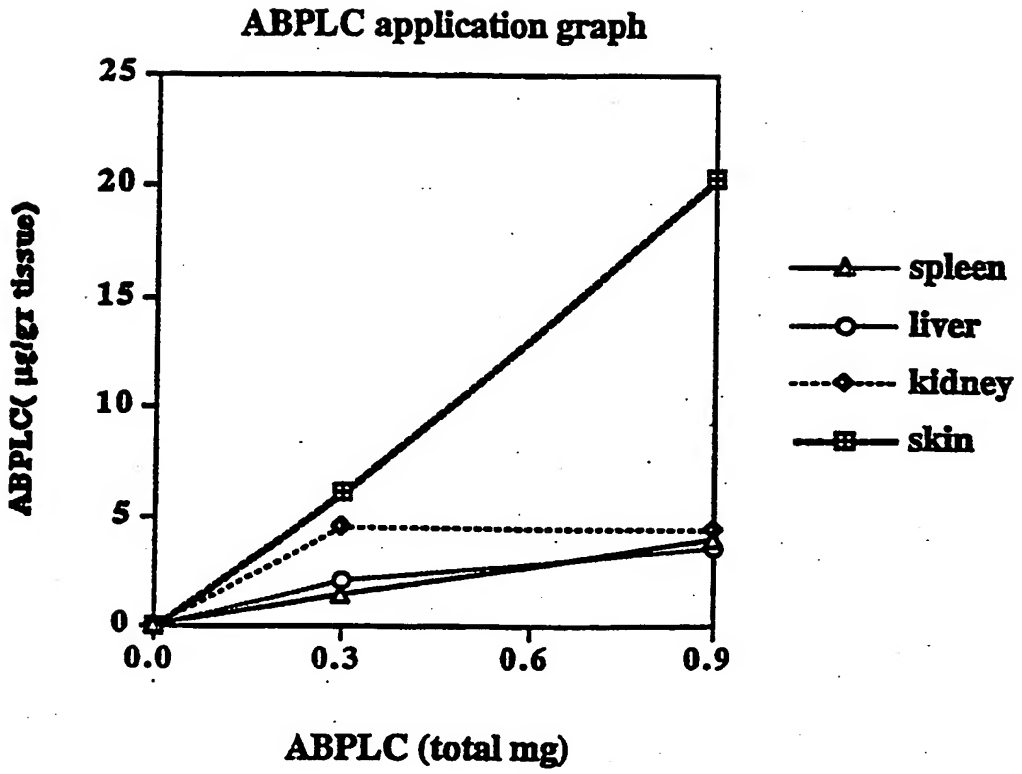
ABPLC application graph
ABPLC(µg/gr tissue)
25
20
15
10
5
0
0.0
0.3
0.6
0.9
ABPLC (total mg)
spleen
liver
kidney
skin

Fig. 5

INTERNATIONAL SEARCH REPORT

Inte... ...al Application No
PCT/IL 98/00582

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K31/70 A61K47/24 A61K47/28 A61K47/48

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5 277 914 A (F.C.SZOKA) 11 January 1994 see claims 1,2,6,7,9,11 ---	1-29
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Y	WO 90 06775 A (LIPOSOME TECHNOLOGY) 28 June 1990 see claims 1-5,18-22 ---	1-29
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
4 March 1999	15/03/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Scarponi, U

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INTERNATIONAL SEARCH REPORT

Inte. ial Application No
PCT/IL 98/00582

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	CHEMICAL ABSTRACTS, vol. 125, no. 2, 8 July 1996 Columbus, Ohio, US; abstract no. 18740, XP002095239 see abstract & J.W.HIEMENZ ET AL.: "LIPID FORMULATIONS OF AMPHOTERICIN B : RECENT PROGRESS AND FUTURE DIRECTIONS" CLIN. INFECT. DIS., vol. 22, no. 2, 1996, pages S133-S144,	1-29
A	EP 0 429 248 A (SHISEIDO) 29 May 1991 see claims see example 10	1-29
A	EP 0 418 153 A (MEDGENIX) 20 March 1991 see claims	1-29
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4

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IL 98/00582

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claim(s) 14 -29
is(are) directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int... ...nal Application No
PCT/IL 98/00582

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